

Immunohistochemical Expression of MMP2, VEGF and D2-40 as Biological Markers of Local Invasion Potential, Angiogenesis and Lymphangiogenesis in Oral Squamous Cell Carcinoma and Verrucous Carcinoma

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ABSTRACT

Background: Verrucous carcinoma (VSCC) is considered as a rare well differentiated carcinoma variant of SCC with no metastatic potential. The aim of this study is to evaluate the Immunohistochemical expression MMP-2, VEGF and D2-40 expression in OSCC and VSCC.

Materials and methods: Thirty formalin fixed paraffin embedded tissue blocks of OSCC and another twelve VSCC were collected and Four micrometer thick sections were cut from each block and mounted on positively charged slides and stained immunohistochemically with monoclonal antibodies to MMP-2, VEGF and D2-40.

Results: there is no statistical difference between SCC and VSCC regarding the immunoexpression of MMP-2 and VEGF. While the lymphatic vessels density were higher in SCC than VSCC

Conclusions: OSCC and VSCC bear similar local invasion and angiogenesis potentials when quantified with MMP-2 and VEGF immunostaining respectively whereas OSCC have higher lymphatic vessels density than their VSCC as evaluated with D2-40 immunoexpression

Keywords: Squamous cell carcinoma, verrucous squamous cell carcinoma, MMP-2, VEGF, D2-40. (J Bagh Coll Dentistry 2016; 28(3):59-64).

INTRODUCTION

Squamous cell carcinoma (SCC) estimated to constitute approximately 94% of all oral malignancies ⁽¹⁾. SCC affects men more than women, usually in the middle to later decades of life. Wide range of tumor features, including size and site, histologic malignant grade, perineural spread at the invasive front, lymphovascular invasion and tumor thickness have been described as major risk factors that adversely affect the prognosis for patients with oral SCC ⁽²⁾.

Throughout the twentieth century this large set of head and neck SCC (HSCC) was clinically considered to be a rather uniform group; their worldwide variations in incidence and anatomic distribution were overwhelmingly attributed to demographic differences in the habits of exposure to smoking or chewing tobacco and drinking alcohol ⁽¹⁾. With the advent of the twenty -first century this view has evolved because refinements of the molecular gene-technique are allowing for the recognition of new subtypes of HNSCC that differ not only in etiology, but also in pathogenesis and clinical outcome ⁽³⁾.

Oral verrucous Squamous Carcinoma (OVSCC) is considered a rare, low-grade and well-differentiated carcinoma, with less potential for lymph node metastasis than other oral carcinomas.

OVC is also called 'Ackerman's tumor' or 'verrucous carcinoma of Ackerman' since it was first reported and described by Ackerman in 1948. VSCC, which accounts for 2.2-20% of all oral cancer, is mainly found in elderly males, particularly in tobacco smokers ⁽⁴⁾. OVC is regarded as a variant of squamous cell carcinoma with specific clinical, pathological and cytokinetic features, which also renders it different from squamous cell carcinoma ⁽⁵⁾. VSCC is probably one of the most difficult and problematic lesions to diagnose in almost every instance. This is because the lesion is not cytologically malignant and therefore evidence of invasion is required for definite diagnosis ⁽⁶⁾.

Local invasion and distant metastases are the most important determining factors in the prognosis of malignant tumors. Degradation of extracellular matrix (ECM) that surrounds tumor cells is one of the essential steps in tumor invasion and the development of metastasis ⁽⁷⁾. MMP-2, matrix metalloproteinase 2, also known as gelatinase A or type IV collagenase, is the most widely expressed of all the MMPs and is found in most tissues and cells ⁽⁸⁾.

Angiogenesis has been shown in experimental animal models to be a crucial step in the successful growth, invasion and metastasis of the tumor. Tumors will not grow beyond 2-3 mm in volume unless an intratumoral capillary network is constructed. Up-regulation of VEGF in tumors is therefore expected to be associated with increased angiogenesis and poor prognosis. However, the association between VEGF

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expression and prognosis, vascularity and disease progression in oral squamous cell carcinoma has not been fully addressed⁽⁹⁾.

Lymphangiogenesis which is the formation of lymphatic vessels from pre-existing one; play an important physiological role in homeostasis, metabolism and immunity. Lymphatic vessel formation has also been implicated in a number of pathological conditions including neoplasm metastasis. Recent evidence suggests an active role of malignant tumors in the induction of intratumoral and peritumorally morphangiogenesis⁽¹⁰⁾. Immunohistochemical markers specific for lymph vessel endothelium have been established recently, among the various antibodies specific for the endothelial cells of lymph vessels but do not stain vascular endothelial cells is the anti D2-40 monoclonal antibody which is relatively widely used⁽¹¹⁾. Identification of lymphatic infiltration of tumor cells with D2-40 monoclonal antibody might make an objective and precise diagnosis of lymphatic metastasis⁽¹²⁾.

Because of the obscure and variable biological behavior of oral VSCC this study will consider different aspects of tumor dynamics such as the invasion potential, angiogenesis and lymphangiogenesis through the immunohistochemical evaluation of the MMP-2, VEGF and D2-40 biological markers respectively in OSCC and VSCC.

MATERIALS AND METHODS

Thirty formalin fixed paraffin embedded tissue blocks which have been diagnosed as OSCC dated from (2000-2012) and twelve formalin-fixed, paraffin-embedded tissue blocks, which have been diagnosed as VSCC dated from (1974 - 2012). Collected from the archives of the department of Oral & Maxillofacial Pathology/ College of Dentistry/ University of Baghdad; Al-Shaheed Ghazi Hospital/Medical City/ Baghdad; Al-Kadhimiya teaching hospital and private laboratories archives.

Diagnostic confirmation was performed through examination of hematoxylin and eosin (H&E) stained sections. Four micrometer thick sections were cut and mounted on positively charged slides and stained immunohistochemically with monoclonal antibodies to MMP-2 to assess the local invasion, VEGF to assess the angiogenic potential and D2-40 to assess the lymphangiogenic potential.

Comparison regarding aforementioned markers expressions was carried out between the two tumors involved in the study.

RESULTS

The age range of the patients with oral squamous cell carcinoma was between 24 and 99 years with a mean of (59.23±14.96). For verrucous carcinoma the age ranged between 32 and 83 years with mean of (59.83 ±15.33), with no significant statistical difference in the age distribution between the two groups. Theme: female ratio for squamous cell carcinoma was 18/12 (1.5:1) and it was 9/3(3:1) for verrucous carcinoma.

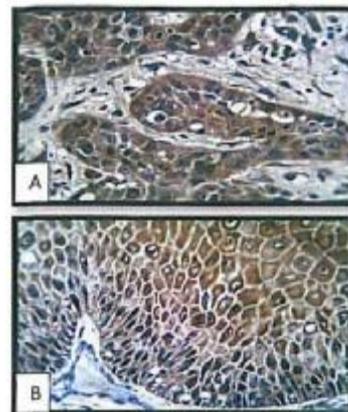
No statistically significant difference was found regarding sex distribution between the groups. Regarding the location, the tongue represented the most predominant site (26.7%) for squamous cell carcinoma and in verrucous squamous carcinoma cases the lower ridge and buccal mucosa showed similar frequency 4 (33.3%).

Histological examination showed that 15 cases (50%) of squamous cell carcinoma were well differentiated, followed by 11 cases (36.7%) were moderately differentiated and 4 cases (33.3%) were poorly differentiated. MMP-2 immunoreactivity was recognized in 28 (93.3%) of squamous cell carcinoma cases.

In verrucous squamous carcinoma all cases (100%) showed positive immunoreactivity with no statistically significant difference in its expression between squamous cell carcinoma and verrucous squamous carcinoma (table-1).

Table 1: MMP-2 scores in OSCC and VSCC

MMP-2 score	OSCC		VSCC	
	Frequency	Percent	Frequency	Percent
Score 0	2	6.7%	0	0%
Score 1	10	33.3%	1	8.3%
Score 2	9	30.0%	4	33.3%
Score 3	9	30.0%	7	58.3%
Total	30	100%	12	100%
Test	Chi-square 0.19 NS			



**Figure 1: MMP-2 immunoreactivity in A. SCC
B. VSCC**

Collectively, 35 of 42 cases (83.3%) were positive for VEGF antibody with different score value. Concerning the squamous cell carcinoma, 23 of 30 (76.7%) cases were positive to VEGF antibody, whereas there were no VEGF negative verrucous squamous carcinoma cases. No statistically significant difference in the VEGF immune expression between the two groups (table-2).

Table 2: VEGF score in OSCC and VSCC

VEGF score	SCC		VSCC	
	Frequency	Percent	frequency	Percent
Score 0	7	23.3%	0	0%
Score 1	10	33.3%	3	25%
Score 2	5	16.7%	5	41.7%
Score 3	8	26.7%	4	33.3%
Total	30	100%	12	100%
Test		Chi-square 0.146 NS		

D2-40 immunostaining revealed positive lymphatic vessels immunoreactivity in all cases of squamous cell carcinoma. In verrucous squamous carcinoma 5 cases showed positive intra- tumoral lymphatic vessels density (41.6%), and 7 cases peritumoral lymphatic vessels density (58.33%). There was statistically significant difference in the lymphatic vessels density in all parameters (intra-tumoral, peritumoral and total lymphatic vessels) between the two groups (table- 3). Lymphatic vessel invasion was detected in 19 (63%) of squamous cell carcinoma cases, it was not seen in any cases of verrucous carcinoma cases. Tumor cells positivity to D2-40 antibody was recognized in 25 cases (83.3 %) of squamous cell carcinoma cases, and 10 cases (83.3%) of verrucous carcinoma cases .No statistically significant difference between the two groups (table-4).

Table 3: Mean labeling index of the ILVD, PLVD and TLVD in OSCC and VSCC

	SCC	VSCC	t-Test
ILVD	14.3±8.3	2.16±2.48	0.00**
PLVD	12.13±5.6	6.2±3.5	0.005*
TLVD	26.43 ± 12.83	8.42 ± 5.52	0.0005*

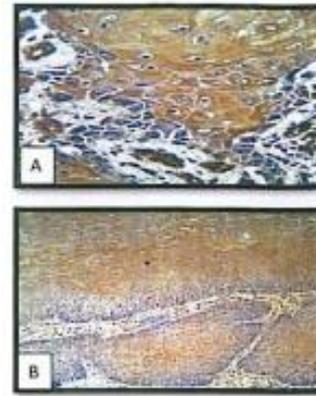


Figure 2: VEGF immunorexpression in A. SCC B. VSCC

Table 4: D2-40 tumor cell scores in OSCC and VSCC

D2-40 score	SCC		VSCC	
	Frequency	Percent	Frequency	Percent
Score 0	5	16.7%	2	16.7%
Score 1	12	40%	2	16.7%
Score 2	12	40%	6	50%
Score 3	1	3.3%	2	16.7%
Total	30	100%	12	100%
Test		Chi-square 0.97 NS		

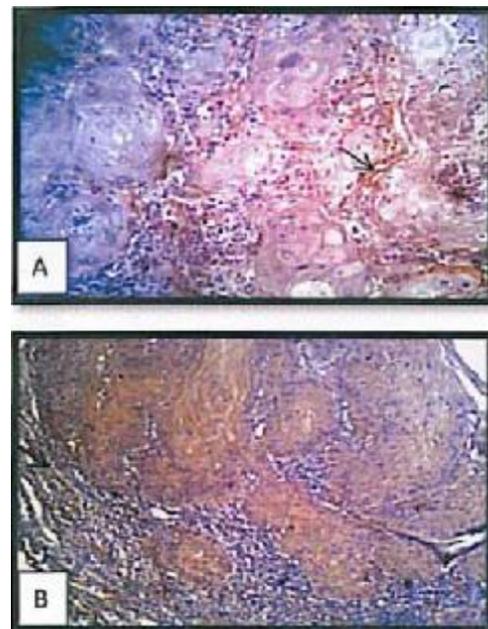


Figure 3: D2-40 immunorexpression in A. SCC B. VSCC

DISCUSSION

Assessment of MMP-2 immunostaining

The role played by MMPs in the progression of oral cancer appears increasingly interesting⁽¹³⁾ Cells initially penetrate the basement membrane and migrate through the stoma. Thus proteolysis of extracellular matrix macromolecules is a

crucial step in cancer invasion and metastasis. The cancer cells produce different extracellular matrix degrading enzymes, one of them is MMP-2, which play a role in the malignant behavior of the neoplasm⁽¹⁴⁾.

In this study 28(93.3%) of OSCC cases were MMP-2 positive and (35.7%) of this positive cases showed low expression, this finding is near the finding of Ruokolainen et al,⁽¹⁵⁾ who found MMP-2 expressed in (89%), while Vicente et al⁽¹⁶⁾ found MMP-2 in 32% of oral SCC patients. In VSCC cases MMP-2 expression was found in all cases (100%) and (8.3%) of cases were weak expression.

Although there is no statistical significant different between the expression of MMP-2 in VSCC and OSCC but the expression was higher in VSCC. This agrees with Tang et al⁽⁷⁾ who used RT-PCR to test MMP-2 in OSCC and VC and found that the expression of MMP2 mRNA in verrucous carcinoma was significantly higher than that in well- differentiated and moderately or poorly differentiated squamous cell carcinoma. This increased in MMP-2 expression in VSCC may explain the aggressive local invasion of these tumors. The variations seen in the immunopositivity rate among different studies could be due to the antibodies used and the processing techniques performed, as well as the heterogeneity of OSCC which could be attributed to the epidemiological or biological differences between countries and population. Besides, the production of MMPs by normal cell is regulated by growth factors and/or cytokines, therefore heterogeneity also could be due to the expression of specific receptors for these enzymes⁽¹⁸⁾

Assessment of VEGF Immunostaining

VEGF is known to be one of the most pivotal angiogenic factors responsible for inducing tumor angiogenesis since it is the only angiogenic peptide known to act specifically on endothelial cell, and it is therefore considered as a leading candidate of angiogenesis⁽¹⁹⁾.

This study showed a positive expression to VEGF antibody in 35cases (83.3%), 23 cases of OSCC (76.7%) while all VSCC cases were positive to different extents. In agreement with this study, Sarkis et al,⁽²⁰⁾ and Mărgăritescu et al⁽²¹⁾ recorded (100%), (87.5%) and (87%) of OSCC cases expressed VEGF immunostaining respectively. The high expression of VEGF may be explained by several points. Tumor cells may produce VEGF not only for vessel sprouting, but also to use it as an autocrine growth factor. Some studies have demonstrated the existence of VEGF receptors in oral SCC cells⁽²²⁾ suggesting an

autocrine role of VEGF. MMPs Cleavages type IV collagen of the epithelial and vascular basement membrane also stimulates release of VEGF from ECM-sequestered pools⁽²³⁾

Assessment of D2-40 Immunostaining: Assessment of ITLVD and PTLVD

D2-40 positive lymphatic vessels were detected in all cases of SCC both peritumorally and intra-tumorally. while positive intratumoral lymphatic vessels were detected in only 5 cases of the VSCC (41.6%), and peritumorallymphatic vessels in 7 cases (58.33%). There is a significant difference in LVD between SCC and VSCC the mean of TLVD in SCC (26.43 ± 12.83) while in VSCC (8.42 ± 5.52). The high rate of LVD inSCC than VSCC may explain the high ability of SCC to metastasis and it is evident that lymphangiogenesis is an important feature of SCC.

Assessment of LVI

The invasion of tumor cells into LVs is one of the critical steps for the establishment of metastasis⁽²⁴⁾ LVI has been included as a new risk factor for cancer patients and its presence might be a predictor for postoperative prognosis⁽²⁵⁾ According to the results of the present study, D2-40 immunostaining highlighted the presence of LVI in 19 D2-40 positive lymphatic vessel cases (63.3%) out of 30 cases of squamous cell carcinoma. While LVI not found in any case of verrucous carcinoma.

Assessment of D2-40Expression in Tumor Cells

The positivity of tumor cells was detected as membranous and /or cytoplasmic localization. The distribution of immunostaining in tumor cells revealed D2-40 expression in 25 cases (83.3 %) of OSCC and in VSCC it expression in 10 cases (83.3%).There is no significant difference between two groups. Sarkis et al.⁽²⁰⁾ detected positive tumor cells as membranous and/or cytoplasmic localization in 15(37.5%) cases of SCC whereas normal oral mucosal cells showed no immunoreactivity.

Abdullah⁽²⁶⁾ found diffuse positive immunohistochemical staining in all verrucous carcinoma tissue samples extending from surface epithelium to the basal layer while in squamous cell papilloma only the basal cell layer show positive immunoreactivity. Similarly, Taher et al.⁽²⁷⁾ found that D240 was expressed in malignant squamous cells of

mucoepidermoid carcinomas unlike the mucous cells. From the above findings it is possible to assume that D2-40 may be a useful marker for distinguishing malignant neoplasms from benign epithelial neoplasms, moreover its expression may act as a good tumor marker in the differential diagnosis of certain carcinomas from their potential histologic mimics since the presence of morphological similarities between the cells of some neoplastic lesions and their normal or benign counterparts impose diagnostic difficulties. In conclusion it seems that LVD is a crucial point in determining the metastatic potential of SCC in comparison to VSCC which showed a significant difference in ILVD, PLVD and TLVD.

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